

Molecular Characterization of the *Fusarium graminearum* Species Complex in Japan

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ABSTRACT

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Members of the *Fusarium graminearum* species complex are important cereal pathogens worldwide and belong to one of at least nine phylogenetically distinct species. We examined 298 strains of the *F. graminearum* species complex collected from wheat or barley in Japan to determine the species and trichothecene chemotype. Phylogenetic analyses and species-diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLPs) revealed the presence and differential distribution of *F. graminearum sensu stricto* (s. str.) and *F. asiaticum*

in Japan. *F. graminearum* s. str. is predominant in the north, especially in the Hokkaido area, while *F. asiaticum* is predominant in southern regions. In the Tohoku area, these species co-occurred. Trichothecene chemotyping of all strains by multiplex PCR revealed significantly different chemotype compositions of these species. All 50 strains of *F. graminearum* s. str. were of a 15- or 3-acetyl deoxynivalenol type, while 173 (70%) out of 246 strains of *F. asiaticum* were of a nivalenol type. The possibility of gene flow between the two species was investigated by use of 15 PCR-RFLP markers developed in this study. However, no obvious hybrids were detected from 98 strains examined, including strains collected from regions where both species co-occur.

Additional keywords: Fusarium head blight, scab.

Fusarium graminearum Schwabe (teleomorph: *Gibberella zeae*) is an important cereal pathogen worldwide. The fungus causes Fusarium head blight (FHB) or scab of wheat, barley and rice, and ear rot of maize. In addition to quantitative yield losses, harvested grain sustains qualitative problems of contamination with mycotoxins such as nivalenol (NIV), deoxynivalenol (DON), and zearalenone. In Japan, about half of the wheat yield was estimated to have been lost to this disease in 1963 and more recently, considerable losses occurred in 1998 and 1999 (2). A total of 1.3 billion dollars was estimated to have been lost due to the same disease between 1991 and 1997 in the United States (6).

Many *Fusarium* species and *Microdochium nivale* (Fries) Samuels and Hallett are associated with FHB (10). *F. graminearum* was the most frequently isolated pathogen in Japan (9). Molecular phylogenetic analyses using world wide collections of *F. graminearum* revealed that *F. graminearum* is a species complex (*F. graminearum* complex) consisting of at least nine biogeographically structured species (17,18,28). *F. graminearum* s. str. (previously lineage 7) is the dominant *F. graminearum* complex

species associated with head blight in North America, whereas *F. asiaticum* (previously lineage 6) appears to be the major species in temperate regions of Asia. Two Japanese strains were examined in previous molecular phylogenetic analyses, and both were identified as *F. asiaticum*. Further investigations of a larger number of strains are needed to refine the knowledge of the distribution of the nine species in order to inform plant quarantine programs aimed at preventing the introduction of foreign FHB species.

Since morphological differences could not be found between *F. graminearum* s. str. and *F. asiaticum* (18), diagnostic methods based on genome information became essential for large surveys of these species. Molecular phylogenetic analysis based on amplified fragment length polymorphism (AFLP) data was used to document the presence of *F. graminearum* s. str. and *F. cortaderiae* in New Zealand (13). Although automated AFLP analysis is a powerful tool to investigate a large number of strains, representative strains of all nine species are always required for species identification. Therefore, we developed a diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) system for *F. graminearum* s. str. and *F. asiaticum* based on fixed nucleotide polymorphisms between the species to investigate Japanese strains of the *F. graminearum* complex.

Information on the occurrence of hybrids as well as the distribution of the nine species is important in understanding fungal evolution. Successful interspecific crossing in vitro has been reported in the *F. graminearum* complex (3,7). However, beyond a single hybrid between *F. meridionale* and *F. asiaticum* that was

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*The e-Xtra logo stands for "electronic extra" and indicates that a supplemental table showing strain history information and results of molecular characterization is available online.

detected from Nepal (17) the natural occurrence of interspecific hybrids is undocumented. Reciprocal monophyly of the nine species within the *F. graminearum* complex is unlikely to be inferred if interspecific hybridization were common enough in nature to allow homogenization of the species. No hybrids between *F. graminearum* s. str. and *F. cortaderiae* were detected in New Zealand where these species co-occur (13).

Individual *F. graminearum* complex isolates have tendencies to produce different trichothecene profiles (chemotype). Some strains mainly produce deoxynivalenol (DON) content and 3-acetyl deoxynivalenol (3ADON), some mainly produce DON and 15-acetyl deoxynivalenol (15ADON), and some mainly produce NIV and acetylated derivatives (11). Ward et al (28) demonstrated that reciprocal monophyly of these three chemotype in *Tri3*, *Tri11*, and *Tri12* gene trees and developed a chemotype specific PCR assay. These chemotypes may affect species or population ecology as oxygenation or acetylation is known to alter the toxicity and bioactivity of trichothecenes (1,8). All three trichothecene chemotypes have been found in *F. graminearum* s. str. (28). However, regional differences of trichothecene chemotype composition may be present in some species. In a small survey of New Zealand FHB, the 3ADON type was not detected from 15 strains of *F. graminearum* s. str. and all 13 strains of *F. cortaderiae* had the NIV chemotype (13). In Japan, DON and NIV producing strains were found to be distributed differently (29). However, the association between trichothecene chemotype and species composition among *F. graminearum* complex strains from Japan is unknown.

In this study, we investigated the species distribution and trichothecene chemotype composition of Japanese strains of the *F. graminearum* complex and the possibility of natural hybridization between *F. graminearum* s. str. and *F. asiaticum* in Japan.

MATERIALS AND METHODS

Fungal strains. Strains were collected from symptomatic wheat or barley heads harvested from 2001 to 2004 in 35 prefectures in Japan (Fig. 1).

Most strains were isolated as follows, but some strains were also provided by collaborators. Salmon pink colored sporodochia on heads were plated on water agar or potato sucrose agar (PSA) containing 300 µg of streptomycin medium per ml. A single conidium was then subcultured on PSA. Heads without sporodochia were kept in a humidity chamber (>95% relative humidity) for further growth or placed on *F. graminearum*-selective medium modified from Komada's medium (25). Colonies were subcultured on synthetic nutrient agar (SNA) for conidiation. Conidia produced on SNA were spread on water agar and a single conidium was isolated. Colonies showing typical morphologies for *F. graminearum* on PSA, such as red pigmentation and fast growth were used in this study. Colonies with uncertain morphologies for *F. graminearum* on PSA were subcultured on SNA and macroconidia were observed microscopically. A total of 298 strains were obtained from different heads. Each strain was given a unique seven digit number: the first two denote the year that seeds were harvested, followed by a number for the prefecture (Fig. 1), and a number within a prefecture. For example, strain 0301201 was isolated from the Hokkaido prefecture in 2003.

Genomic DNA extraction. Each strain was cultured in potato dextrose broth. The resulting mycelial mat was air-dried and thereafter ground to a powder by vortexing with small pieces of stainless steel wire in a microtube. The mycelial powder was mixed with 300 µl of potassium ethyl xanthogenate (PEX) solution (6.25 mM potassium ethyl xantogenate, 100 mM Tris-HCl, pH 7.5, 700 mM NaCl, and 10 mM EDTA, pH 8.0), and incubated at 65°C for 30 min (14). The microtube was centrifuged at 12,000 × g for 5 min. An aliquot of 160 µl of the solution was transferred to a new microtube and mixed with 400 µl of ethanol. DNA was precipitated by centrifugation at 12,000 × g for 5 min. After rinsing with 70% ethanol and drying, the DNA pellet was resuspended in 400 µl of water. The concentration of genomic DNA was estimated to be about 5 ng/µl by comparison to DNA of known concentration using agarose gel electrophoresis.

PCR and sequencing. A portion of the reductase and the histone H3 genes were amplified by PCR and sequenced accord-

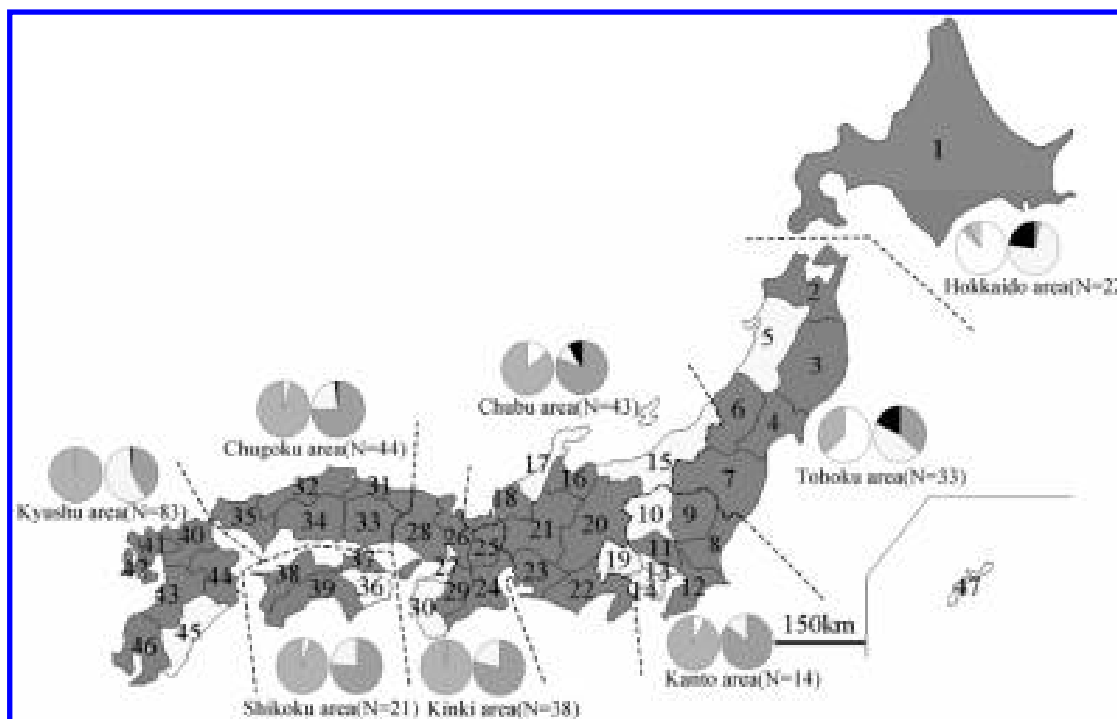


Fig. 1. Prefectures from which strains of the *Fusarium graminearum* species complex were collected in Japan are shown in gray. The numbers correspond to the prefecture information presented in Table 2. **A**, Left pie chart shows species composition in each area; white, gray, and cross stripes indicate *F. graminearum* s. str., *F. asiaticum*, and the unknown species, respectively. **B**, Right pie chart shows chemotype composition in each area; hatched, spot, and black indicate nivalenol, 3-acetyl deoxynivalenol and 15-acetyl deoxynivalenol type, respectively.

ing to O'Donnell et al. (17). PCR was performed in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA), using the following cycling parameters: 94°C for 2 min, 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. After PCR, a 0.6 volume of polyethylene glycol (PEG) solution (30% polyethylene glycol 6000, 1.6 M NaCl) was added and then incubated at 37°C for 10 min. DNA was precipitated by centrifugation (12,000 × g, 10 min) and rinsed with 70% ethanol. After drying, the DNA pellet was resuspended in water and used for sequencing. Sequencing reactions were carried out with BigDye terminator V3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA) and then run on an ABI 3100 genetic analyzer (Applied Biosystems). DNA sequences were edited and aligned using Sequencer version 3.1.1 (Gene Codes, Ann Arbor, MI). Sequences have been deposited under GenBank Accession nos. DQ925702 to DQ925735. *F. graminearum* complex-specific PCR was performed with the UBC85F410 and UBC85R410 primer pair according to Schilling et al. (21).

Phylogenetic analysis. Additional sequence data from strains that belong to nine species of the *F. graminearum* complex and other *Fusarium* spp. were obtained from O'Donnell et al. (18). All sequences were aligned using CLUSTAL X (24). Ambiguously aligned positions were removed using the Se-Al alignment editor (19). Sequences of the reductase and histone H3 genes were combined (1,225 bp) and used for phylogenetic analysis conducted with PAUP* 4.0 (23). Maximum parsimony analysis used a heuristic search with simple sequence addition and tree bisection with reconnection branch swapping. Clade stability was assessed by 1,000 bootstrap replications with MAXTREES set to automatically increase. All nucleotide substitutions were equally weighted and unordered. Alignment gaps were treated as missing data.

Diagnostic PCR-RFLPs for *F. asiaticum* and *F. graminearum* s. str. The H3dStyI primer (5'-AGCATCACCYGAACATCGCATCATCCCATG-3') was designed with dCAPS (derived cleaved amplified polymorphic sequence analysis) Finder 2.0 (15). A part of the histone H3 gene was amplified by PCR using primers H3dStyI and H3R1 (5'-TTGGACTGGATRGTAACACGC-3') (18). The total volume of the reaction mixture was 20 µl containing 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (wt/vol) gelatin, 200 µM dNTP, 0.5 µM each primer, 0.025 units of Taq polymerase, and 5 ng/µl of genomic DNA. Cycling parameters were: 94°C for 2 min, 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The PCR amplicon of 223 bp was confirmed by using 5 µl of the PCR mixture for 1% agarose gel electrophoresis. The 100-bp ladder (New England Biolabs, Beverly, MA) was used as size standard. The remaining 7 µl of the PCR mixture was used for digestion with 0.1 unit of StyI (Nippon Gene, Tokyo, Japan) or EcoRV (New England Biolabs) in a 15-µl reaction mixture. The mixtures were incubated for 1 h and then used for electrophoresis using 2% Metaphor agarose (Cambrex Bio Science, Rockland, ME) and 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) (20). Gels were stained in 0.5 µg/ml of ethidium bromide and photographed by Imagemaster VDS (Amersham Pharmacia Biotech, Uppsala, Sweden). NRRL26156 and NRRL31084 were used as representative strains of *F. asiaticum* and *F. graminearum* s. str., respectively. The PCR amplicon from *F. asiaticum* was cleaved into 195 and 28 bp with StyI, while the amplicon from *F. graminearum* s. str. was cleaved into 191 and 32 bp with EcoRV.

Chemotype-specific multiplex PCR. Trichothecene chemotypes were determined by two sets of multiplex PCR based on *Tri3* and *Tri12* sequences (28). The primers used in the *Tri3* multiplex included 3CON (5'-TGGCAAAGACTGGTTCAC-3'), 3NA (5'-GTGCACAGAATATACGAGC-3'), 3D15A (5'-ACTGACCCAAGCTGCCATC-3'), and 3D3A (5'-CGCATTTGGCTAACACATG-3'). The primers used in the *Tri12* multiplex included 12CON (5'-CATGAGCATGGTGATGTC-3'), 12NF (5'-TCTC-

CTCGTTGTATCTGG-3'), 12-15F (5'-TACAGCGGTCGCAAC-TTC-3'), and 12-3F (5'-CTTTGGCAAGCCCGTGCA-3'). Both reactions were performed in 10 µl with 2 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate, and 0.2 µM each primer. PCR consisted of an initial denaturation of 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C. PCR amplicons were separated on 1% agarose gels. Chemotyping by the *Tri3* multiplex PCR was assessed by the following amplicons: 840, 610, and 243 bp for NIV, 15ADON, and 3ADON types, respectively. Chemotyping by the *Tri12* multiplex PCR was assessed by the following amplicons: 840, 670, and 410 bp for NIV, 15ADON, and 3ADON types, respectively.

Trichothecene mycotoxin analysis. Nine strains that were determined as being different chemotypes of *F. graminearum* s. str. and *F. asiaticum* by the chemotype-specific multiplex PCR were cultured on sterile rice. Thirty grams of rice grain was soaked in 15 ml of water prior to autoclaving in Erlenmeyer flasks. Each flask was inoculated with three pieces of culture grown on PDA. Each flask was shaken once a day for the first 3 days and several times during the remaining days, and were kept up to 2 weeks after inoculation at 25 to 27°C. Samples were ground and then sonicated in 200 ml of acetonitrile/water (85:5, vol/vol). After treatment by the multifunctional column, MultiSep#227 (Romer Labs, Union, MO), trichothecenes were quantified by liquid chromatography-mass spectrometry (Model ZQ Quattro Micro, Waters, Milford, MA) with an octadecyl silica column and the mobile phase of acetonitrile/10 mM ammonia acetate water/methanol (5:90:5) for DON or NIV and with four serial octadecyl silica columns and acetonitrile/10 mM ammonia acetate water (25:75) for 3ADON or 15ADON. Each mycotoxin was confirmed by gas chromatography-mass spectrometry (Model 5973 inert MSD, Agilent Technologies, Palo Alto, CA) with a DB-35ms column (Agilent Technologies) and helium gas after trimethylsilylation. Standards for DON, 3ADON, 15ADON, and NIV were obtained from Sigma Aldrich (Chicago, IL). The detection limit for each mycotoxin was 0.05 µg/kg.

Marker development for detection of hybrids between *F. graminearum* s. str. and *F. asiaticum*. Primer pairs designed from expressed sequence tags (26) were used for the marker development. PCR was performed as described above with cycling parameters being set at 94°C for 2 min, 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. A single restriction enzyme (*Hinf*I, New England Biolabs, Beverly, MA) was used for the selection of primer pairs in order to simultaneously collect all data. Fifteen primer pairs out of 352 pairs tested gave a single amplicon in addition to distinct polymorphisms between strain 0101020 of *F. graminearum* s. str. and strain 0246002 of *F. asiaticum* after 1% agarose gel electrophoresis. The genomic locations of these markers were determined by a blastn search (Broad Institute of MIT and Harvard) and Gale et al. (5) (Table 1).

RESULTS

***F. graminearum* complex-specific PCR and phylogenetic analyses.** We used primers UBC85F410 and UBC85R410 (21) for easy identification of the *F. graminearum* complex instead of laborious microscopic observation. These primers amplify a 332-bp amplicon specific to the *F. graminearum* complex (21). The 332-bp amplicon was observed for 294 out of the 298 strains although several extra bands were also usually amplified. The 332-bp amplicon was missing from four strains; 0403016, 0407141, 0231001, and 0232003. Their cultural morphologies were indistinguishable from the other strains. Therefore, portions of the reductase and histone H3 genes of these strains were sequenced to clarify whether they belong to the *F. graminearum* complex. Each of these strains contained *F. graminearum* s. str. or *F. asiaticum*-specific nucleotides described by O'Donnell et al. (18), with the exception of an *F. asiaticum*-specific guanine at

position 52 in the reductase gene sequence that was previously reported as adenine by O'Donnell et al. (18).

Phylogenetic analysis of the reductase and histone H3 gene sequences placed strain 0403016 in the *F. graminearum* s. str. clade, while the remaining three strains were placed in the *F. asiaticum* clade (Fig. 2). Ten randomly selected strains that produced the 332-bp amplicon were similarly placed in either the *F. graminearum* s. str. clade (0301201 and 0403001) or the *F. asiaticum* clade (0406021, 0216021, 0421008, 0231009, 0235001, 0237005, 0240013, and 0244018) (Fig. 2). All of the strains that were placed in *F. asiaticum* clade have guanine at position 52 in the reductase gene sequence.

Development of a diagnostic PCR-RFLP for *F. graminearum* s. str. and *F. asiaticum*. A schematic diagram of the diagnostic PCR-RFLP is shown in Figure 3. The dCAPS primer H3dStyI was designed from the histone H3 gene sequence of *F. graminearum* s. str. and *F. asiaticum*. The 3' terminal sequence of H3dStyI corresponds to a guanine at position 278 in histone H3 that is unique to *F. asiaticum* (18) and completes the StyI recognition sequence (CCWWG \underline{G}). Specific StyI-cleavage of the amplicon from *F. asiaticum* was confirmed with representatives of the nine species within the *F. graminearum* complex (Fig. 4A). *F. graminearum* s. str. has a unique nucleotide, thymine, at position 279 in the histone H3 gene (18), which creates an EcoRV recognition sequence (GAT \underline{ATC}). Specific EcoRV-cleavage of amplicons from *F. graminearum* s. str. strains was also confirmed (Fig. 4B).

Species and trichothecene chemotype composition of the *F. graminearum* complex in Japan. The species identity and trichothecene chemotype of 298 strains were investigated by the diagnostic PCR-RFLP method developed for *F. graminearum* s. str. and *F. asiaticum* (Fig. 4) and the chemotype-specific multiplex PCR for *Tri3* and *Tri12*, respectively. PCR amplicons from 245 strains using primers H3dStyI and H3R1 were cleaved by StyI, and therefore they were identified as *F. asiaticum* (Table 2). Fifty strains were identified as *F. graminearum* s. str. based on the fact that the amplicons were cleaved by EcoRV (Table 2). Neither StyI nor EcoRV cleaved the amplicon from strain 0301112, 0301831, and 0239005. Therefore, portions of their reductase and histone H3 genes were sequenced and included in phylogenetic analysis. The histone H3 gene sequence of strain 0239005 revealed that the *F. asiaticum*-specific guanine at position 278 (18) was substituted to adenine. However, all other nucleotide characters unique to *F. asiaticum* in the reductase and

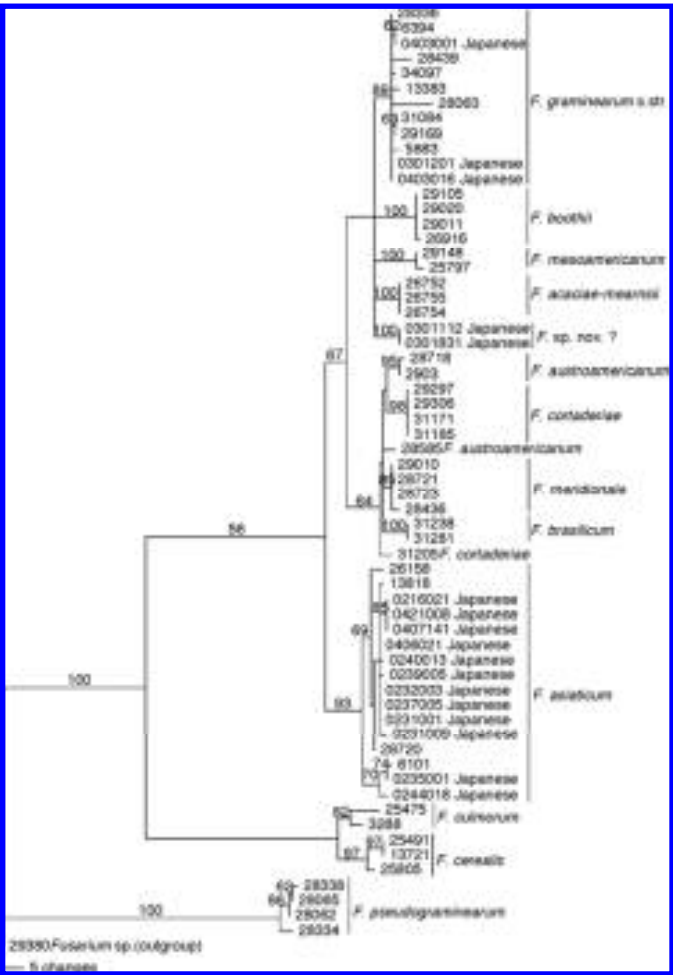


Fig. 2. Phylogenetic trees based on sequences of the reductase and histone H3 genes generated from Japanese strains of the *Fusarium graminearum* species complex. One of two most-parsimonious phylograms inferred from the combined sequence (1,225 bp) of the reductase and the histone H3 gene. The tree length is 276 steps, consistency index = 0.84, and retention index = 0.95. *Fusarium* sp. NRRL29380 was used to root the trees. Bootstrap frequencies are shown above branches recovered in at least 50% of 1,000 bootstrap replicates. All strains except for Japanese strains isolated in this study have an NRRL designation.

TABLE 1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) markers that distinguish *Fusarium graminearum* s. str. and *F. asiaticum*^a

Locus ^b	Primer sequences ^c
HK703/Ch1/Sc1/Ct1.10/112242	F: 5'-TCACGGCTCTCGACTTTTGA-3' R: 5'-AATCAACGCAATCTCGCA-3'
HK379Ch1/Sc1/Ct1.16/105470	F: 5'-CATGGCTTATAACCGTCCCTA-3' R: 5'-ATCCATATGACGTTTCGAGGC-3'
HK249/Ch1/Sc1/Ct1.53/17465	F: 5'-TGCCGTGATAAGCTCAAAGA-3' R: 5'-TCATCCTACCAACCTTCTG-3'
HK345/Ch1/Sc1/Ct1.71/8646	F: 5'-AAACAAATCCCCTCATCGTC-3' R: 5'-AACATGTACAGCGTGATTTGG-3'
HK463/Ch1/Sc1/Ct1.114/69003	F: 5'-CAACATCTGTGGCATACCGTT-3' R: 5'-ATCCCTCTTCAAACACCA-3'
HK145/Ch1/Sc1/Ct1.116/125354 (FG02322.1) ^d	F: 5'-TCGGAGGAGCTTTTACTG-3' R: 5'-GACCTTGACATTCTTCC-3'
HK731/Ch2/Sc2/Ct1.150/165042	F: 5'-CCAGGATTATGAATGCCATGA-3' R: 5'-GGTGATGAGGTATCTGCACT-3'
HK435/Ch2/Sc2/Ct1.152/50847	F: 5'-CCCTCTAAACAAATGCAGCA-3' R: 5'-ACCTCAACCTTCAGAACCC-3'
HK517/Ch2/Sc2/Ct1.159/28153	F: 5'-ATCATCGCGACATTATGCTC-3' R: 5'-ATGAATCTCCCTACCCGAAT-3'
HK757/Ch2/Sc2/Ct1.179/26613	F: 5'-GCACGAGGCAAACAAGTTTA-3' R: 5'-GCGTATCGTATGCTGCAACA-3'
HK253/Ch2/Sc2/Ct1.185/189241	F: 5'-GGGACCCTTCAGTGACATCA-3' R: 5'-TTGGTTTCGAGATGAGATTG-3'
HK293/Ch3/Sc3/Ct1.197/46034	F: 5'-TCTACACCACCATGCCTACA-3' R: 5'-ACGATACCAAGCAGGAAGAGA-3'
HK337/Ch3/Sc8/Ct1.449/31483	F: 5'-TCAGACCACTCCAAACAACC-3' R: 5'-TATATACCCGCCGTTTCTT-3'
HK527/Ch4/Sc4/Ct1.276/26096	F: 5'-TGGTGCTCAGTCGTCTTGGT-3' R: 5'-TACTACGGGAAGCATCATGG-3'
HK271/Ch4/Sc6/Ct1.371/111980	F: 5'-CAGTTCGACCAAAATTCCCA-3' R: 5'-TTCGTCTCATACCTCCTC-3'

^a *Hinf*I digestion of the PCR amplicon displays polymorphism between *F. graminearum* s. str. and *F. asiaticum*. Primer pairs designed from expressed sequence tags (ESTs) (26) were used to develop the markers.

^b Locus name/Chromosome No./Scaffold No./Contig No./Position in contig. Information based on the *F. graminearum* sequencing project (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html) and Gale et al. (5).

^c F, forward primer; R, reverse primer.

^d Only HK145 was not obtained from an EST. Corresponding gene ID of the *F. graminearum* sequencing project is indicated.

histone gene sequences (18) were present in this strain and phylogenetic analysis placed it in the *F. asiaticum* clade (Fig. 2). Therefore, it was classified as *F. asiaticum*. Two additional strains, 0301112 and 0301831 did not have any of the nucleotide characters specific to nine species of the *F. graminearum* complex in the reductase and histone gene sequences (18). The two strains formed a clade separate from previously described species with high bootstrap support (100%) (Fig. 2).

F. graminearum s. str. was frequently isolated in the Hokkaido area (19 out of 22 strains) (Table 2). In the Tohoku area, both *F. graminearum* s. str. and *F. asiaticum* were isolated at similar frequency; 21 strains were *F. graminearum* s. str. and 33 strains were *F. asiaticum* (Table 2). Except for the Nagano prefecture (No. 20 in Fig. 1), *F. asiaticum* was predominant in the Kanto to Kyushu area; 233 out of 243 strains (Table 2). In contrast, all five strains from the Nagano prefecture were *F. graminearum* s. str. (Table 2).

Results of the chemotype-specific multiplex PCR for *Tri3* and *Tri12* were consistent within a strain. Nine strains including different chemotypes of *F. graminearum* s. str. and *F. asiaticum* were selected and the results were compared to the trichothecenes produced on rice grain. Results from both tests were congruent for all nine strains (Table 3). Eight strains that were determined as the 3ADON or 15ADON type by the multiplex PCR produced DON and, correspondingly 3ADON or 15ADON, with five strains also producing trace quantities of the other types of acetyl derivatives (Table 3). Some strains that mainly produced either 3ADON or 15ADON, similarly, have been reported to produce trace quantities of the other types of acetyl derivatives (12,28). The chemotype of all 298 strains was examined by multiplex PCR. The NIV chemotype was not observed among the 50 strains of *F. graminearum* s. str., while the 3ADON and 15ADON chemotypes were observed among 70 and 30% of *F. graminearum* s. str. strains, respectively (Fig. 5). A single strain with a 15ADON chemotype was identified among 246 strains of *F. asiaticum*, while 70 and 29% of *F. asiaticum* strains displayed the NIV and 3ADON chemotypes, respectively (Fig. 5B, Table 2). Strains 0301112 and 0301831, representing a putative new *F. graminearum* clade species, were of the 15ADON type.

Search for hybrids between *F. graminearum* s. str. and *F. asiaticum*. In vitro crosses between strain 0208351 of *F. graminearum* s. str. and 0403015 of *F. asiaticum* was performed according to Bowden and Leslie (3). Individual progeny developed in the crosses showed typical hybrid patterns with 15 PCR-RFLP markers, i.e., some markers displayed *F. graminearum* s. str. patterns while others displayed *F. asiaticum* patterns (data not shown). In contrast, no hybrid patterns were identified in 98 field strains from Japan, including 33 strains from the Tohoku

area where *F. graminearum* s. str. and *F. asiaticum* co-occur (Table 4).

DISCUSSION

We developed in this study diagnostic PCR-RFLPs for *F. graminearum* s. str. and *F. asiaticum* (Fig. 4). Prior to this study, an 86-bp insertion specific to *F. asiaticum* was found in amplicons using primers Fg16F and Fg16R that were originally designed for *F. graminearum* complex-specific PCR (16,27). The specificity of the primer pair was tested on seven species of the *F. graminearum* complex and an amplicon was produced for only four species: *F. austroamericanum*, *F. meridionale*, *F. asiaticum*, and *F. graminearum* s. str. (27). Although intraspecies size variation of the amplicons was observed in *F. asiaticum* and *F. graminearum* s. str., all four strains of *F. asiaticum* had the 86-bp insertion (27). However, not many strains were tested and *F. cortaderiae* and *F. brasilicum* have since been added to the *F. graminearum* complex (18). The specificity of the diagnostic PCR-RFLPs developed in this study was tested on all nine species of the *F. graminearum* complex (Fig. 4). The diagnostic PCR-RFLPs were based on fixed nucleotide character states specific to *F. asiaticum* or *F. graminearum* s. str. in the *F. graminearum* complex (18) and therefore, these diagnostic PCR-RFLPs are

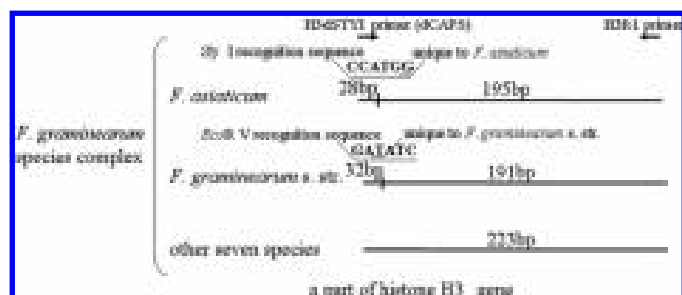


Fig. 3. Schematic diagram of diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for *Fusarium asiaticum* and *F. graminearum* s. str. H3STY1 primer was designed with dCAPS finder (15) in histone H3 gene. A part of the histone H3 gene is amplified by PCR using primers H3STY1 and H3R1. The PCR amplicon from *F. asiaticum* is cleaved into 195 and 28 bp with *StyI*, while the amplicon from *F. graminearum* s. str. is cleaved into 191 and 32 bp with *EcoRV*. Species in the *F. graminearum* complex other than *F. asiaticum* and *F. graminearum* s. str. have neither a *StyI* nor a *EcoRV* site.

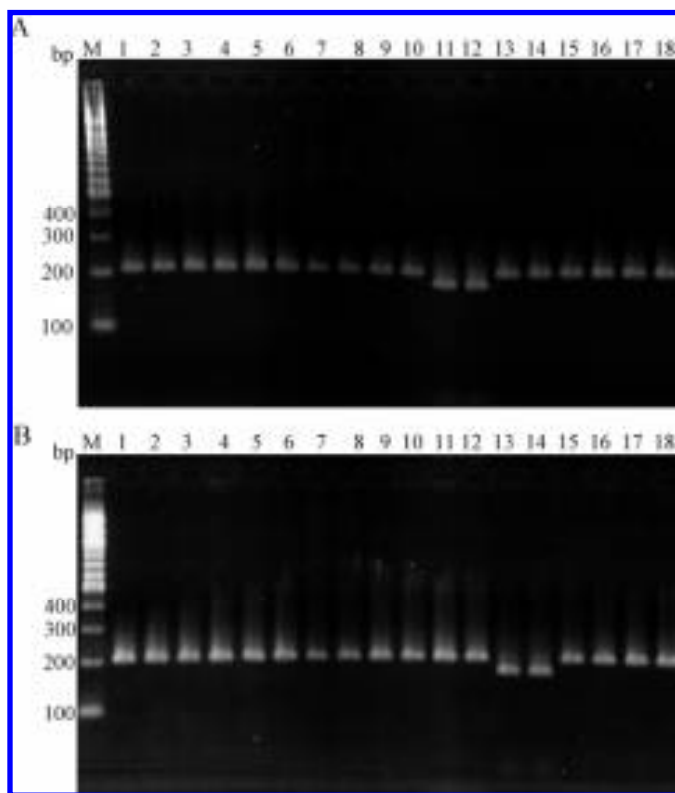


Fig. 4. Diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of *Fusarium asiaticum* and *F. graminearum* s. str. PCR amplicons using primers H3dSTY1 and H3R1 from strains that belong to nine species within the *F. graminearum* species complex were treated with *StyI* (A) or *EcoRV* (B). DNA was separated by electrophoresis in 2% Metaphor agarose (Cambrex Bio Science). Lane M is a 100-bp ladder (New England Biolabs). *F. austroamericanum* strains NRRL2903 (lane 1) and NRRL28585 (lane 2), *F. meridionale* strains NRRL29010 (lane 3) and NRRL28721 (lane 4), *F. boothii* strains NRRL26916 (lane 5) and NRRL29105 (lane 6), *F. mesoamericanum* strains NRRL25797 (lane 7) and NRRL29148 (lane 8), *F. acaciae-meamsii* strains NRRL26755 (lane 9) and NRRL34207 (lane 10), *F. asiaticum* strains NRRL26156 (lane 11) and NRRL6101 (lane 12), *F. graminearum* s. str. strains NRRL31084 (lane 13) and NRRL34097 (lane 14), *F. cortaderiae* strains NRRL29306 (lane 15) and NRRL31205 (lane 16), and *F. brasilicum* strains NRRL31238 (lane 17) and NRRL31281 (lane 18).

applicable only to the members of the *F. graminearum* complex. In this study, all strains were examined by the *F. graminearum* complex-specific PCR (21). Meticulous microscopic observation of a large number of strains is labor-intensive, and not all species

within the *F. graminearum* complex are distinguishable using conidial morphology (18). Therefore, a diagnostic method based on genome information is essential for identification of the species in the *F. graminearum* complex.

TABLE 2. Species identity and trichothecene chemotype composition of *Fusarium graminearum* species complex strains in Japan^a

Area	Prefecture	<i>Fusarium graminearum</i> s. str.				<i>Fusarium asiaticum</i>				Total
		NIV ^b	3ADON	15ADON	Subtotal	NIV	3ADON	15ADON	Subtotal	
Hokkaido	Hokkaido (1) ^c	0	16	3	19	1	0	0	1	20
Tohoku	Aomori (2)	0	3	3	6	0	0	0	0	6
	Iwate (3)	0	5	2	7	4	0	0	4	11
	Miyagi (4)	0	3	0	3	4	0	0	4	7
	Yamagata (6)	0	1	0	1	2	0	0	2	3
	Fukushima (7)	0	3	1	4	2	0	0	2	6
Kanto	Ibaraki (8)	0	1	0	1	4	0	0	4	5
	Tochigi (9)	0	0	0	0	2	0	0	2	2
	Saitama (11)	0	0	0	0	2	0	0	2	2
	Chiba (12)	0	0	0	0	4	1	0	5	5
Chubu	Toyama (16)	0	0	0	0	6	0	0	6	6
	Fukui (18)	0	0	0	0	4	0	0	4	4
	Nagano (20)	0	1	4	5	0	0	0	0	5
	Gifu (21)	0	0	0	0	18	0	0	18	18
	Shizuoka (22)	0	0	0	0	3	1	0	4	4
	Aichi (23)	0	0	1	1	4	1	0	5	6
Kinki	Mie (24)	0	0	0	0	4	2	0	6	6
	Shiga (25)	0	0	0	0	17	2	0	19	19
	Kyoto (26)	0	0	0	0	0	1	0	1	1
	Hyogo (28)	0	0	0	0	5	1	0	6	6
	Nara (29)	0	0	0	0	4	2	0	6	6
Chugoku	Tottori (31)	0	0	0	0	8	1	0	9	9
	Shimane (32)	0	0	0	0	5	1	0	6	6
	Okayama (33)	0	0	0	0	14	2	0	16	16
	Hiroshima (34)	0	1	1	2	3	2	0	5	7
	Yamaguchi (35)	0	0	0	0	3	3	0	6	6
Shikoku	Kagawa (37)	0	0	0	0	6	0	0	6	6
	Ehime (38)	0	1	0	1	8	3	0	11	12
	Kochi (39)	0	0	0	0	2 ^d	1	0	3	3
Kyushu	Fukuoka (40)	0	0	0	0	8	5	0	13	13
	Saga (41)	0	0	0	0	8	8	0	16	16
	Nagasaki (42)	0	0	0	0	4	15	0	19	19
	Kumamoto (43)	0	0	0	0	7	11	1	19	19
	Oita (44)	0	0	0	0	6	7	0	13	13
	Kagoshima (46)	0	0	0	0	1	2	0	3	3
Total		0	35	15	50	173	72	1	246	296 ^e

^a All but four strains were confirmed to belong to the *F. graminearum* species complex by 332-bp polymerase chain reaction (PCR) amplicon specific to the *F. graminearum* species complex (21). Nucleotide sequencing of reductase and histone H3 genes of four strains without the amplicon indicated that they are *F. graminearum* s. str. or *F. asiaticum*.

^b Trichothecene chemotype was determined by chemotype-specific multiplex PCR (28). The number of strains is indicated.

^c The number in parenthesis corresponds to the prefecture number on the map of Japan in Figure 1.

^d Neither *StyI* nor *EcoRV* cleaved the amplicon produced with the H3dStyI and H3R1 primers from strain 0239005. The nucleotide sequences of reductase and histone H3 genes indicated that this strain belongs to *F. asiaticum*, although a target nucleotide of *StyI* cleavage was substituted guanine to adenine.

^e Neither *StyI* nor *EcoRV* cleaved the amplicon produced with the H3dStyI and H3R1 primers from Hokkaido strains 0301112 and 0301831. The nucleotide sequences of the reductase and histone H3 genes suggested they are neither *F. asiaticum* nor *F. graminearum* s. str. Both isolates were of the 15ADON chemotype.

TABLE 3. Trichothecene toxins produced by *Fusarium graminearum* s. str. and *F. asiaticum* in culture on rice grain

Species ^a	Strain	Chemotype ^b	Trichothecene toxins (µg/g) ^c			
			DON	3ADON	15ADON	NIV
<i>F. graminearum</i> s. str.	0101020	3ADON	8.11	5.71	0.33	ND ^d
<i>F. graminearum</i> s. str.	0201001	3ADON	6.33	4.94	0.57	ND
<i>F. graminearum</i> s. str.	0201201	15ADON	12.1	0.11	14.68	ND
<i>F. graminearum</i> s. str.	0402011	15ADON	11.45	0.08	18.78	ND
<i>F. graminearum</i> s. str.	0234007	15ADON	3.15	ND	7.41	ND
<i>F. asiaticum</i>	0228003	3ADON	5.54	1.73	ND	ND
<i>F. asiaticum</i>	0244004	3ADON	0.56	0.30	0.07	ND
<i>F. asiaticum</i>	0239005	NIV	ND	0.05	0.09	1.63
<i>F. asiaticum</i>	0243747	15ADON	5.53	ND	4.21	ND

^a Species determined by the diagnostic polymerase chain reaction(PCR)-restriction fragment length polymorphism based on the histone H3 gene.

^b Chemotype determined by chemotype-specific multiplex PCR (28).

^c Trichothecene toxins produced in culture on rice grain.

^d Less than minimum level of detection (<0.05 µg/g).

Diagnostic PCR-RFLPs indicated that most of the 298 Japanese strains are *F. graminearum* s. str. or *F. asiaticum* but three strains could not be determined. Two of these, 030112 and 0301831, formed a distinct clade in the phylogenetic tree. These strains have recently attained a new species status, *Fusarium vorosii*, based on the analysis of sequences from more genes and morphology (22).

The trichothecene chemotype composition was shown to be significantly different between Japanese isolates of *F. graminearum* s. str. and *F. asiaticum* (Fig. 5). The NIV chemotype was not detected among the 50 *F. graminearum* s. str. strains, while 70% of the *F. asiaticum* strains had the NIV chemotype. Trichothecene chemotype composition also appears to be correlated with geographic origin. It was previously reported that strains with a DON type were predominant in northern areas and Kyushu, whereas strains with an NIV type were predominant in the central area of Japan (29). This could reflect an ecological gradient in Japan that directly influences chemotype distributions. However, differences in the distribution of DON and NIV types may also be explained in terms of the different geographical distributions of *F. graminearum* s. str. and *F. asiaticum*, which differ in their chemotype composition (Fig. 5).

Chemotyping based on genomic information is important since the chemotype is strain-specific and many strains need to be examined to clarify the chemotype composition of each population. Because some strains may not readily produce mycotoxins in vitro, chemotype-specific multiplex PCR tests developed by Ward et al. (28) were used in this study. Results of analyses of trichothecenes produced in culture on a rice medium indicated

that the chemotype can be correctly determined by multiplex PCR (Table 3). Ward et al. (28) previously demonstrated that the *Tri3* and *Tri12* assays could distinguish the NIV, 3ADON, and 15ADON chemotypes of members of the *F. graminearum* complex as well as *F. lunulosporum*, *F. culmorum*, *F. cerealis*, and *F. pseudograminearum*.

While *F. graminearum* s. str. is widely distributed, *F. asiaticum* has its main distribution in Asia (China, Nepal, Japan, and Korea) (18). Both Japanese strains used in previous phylogenetic analyses were *F. asiaticum* (17,18). Results of the phylogenetic analysis (Fig. 2) and diagnostic PCR-RFLPs (Table 2) indicated that *F. graminearum* s. str. is also present in Japan. Furthermore, different geographical distributions of *F. graminearum* s. str. and *F. asiaticum* were revealed in this study. *F. graminearum* s. str. was predominant in northern areas, while *F. asiaticum* was predominant in southern areas (Table 2). In the Chubu area (Nos. 15 to 23 in Fig. 1) where *F. asiaticum* is predominant, all five strains from the Nagano prefecture (No. 20 in Fig. 1) were *F. graminearum* s. str. (Table 2). The mean temperature of Nagano prefecture (12.8°C; at the prefectural capital; 1998 Annual Report, Japan Meteorological Agency) is similar to that of the prefectures in the Tohoku area (10.9 to 13.6°C) rather than those of other prefectures in the Chubu area (14.5 to 16.8°C). Therefore, *F. graminearum* s. str. may be adapted to lower temperatures compared to *F. asiaticum*, although there are some other possible explanations such as differences in relative humidity, cropping practices, etc., and more samples need to be investigated in Nagano prefecture. Nevertheless, no obvious differences in optimum growth temperature have been observed between the two species (T. Aoki, *personal communication*) and no correlation between fungal species (i.e., *F. asiaticum* or *F. graminearum* s. str.) and host origin (i.e., barley or wheat) was identified in this study (data not shown).

F. graminearum s. str. and *F. asiaticum* were isolated in nearly equal frequencies in the Tohoku area (Table 2). Co-occurrence of two species, *F. graminearum* s. str. and *F. cortaderiae*, was also observed in New Zealand (13). Hybrid progeny between *F. graminearum* s. str. and *F. asiaticum* were previously generated by in vitro crosses (7), implying that gene flow may occur between these species in nature and a single, natural hybrid between *F. meridionale* and *F. asiaticum* was isolated in Nepal (17). However, O'Donnell et al. (17,18) concluded on the basis of strong genealogical concordance across multiple loci that hybridization rates among different species of the *F. graminearum* complex have not been frequent enough to counter differentiation by genetic drift. In this study, we could not identify a single hybrid among 98 strains that included 33 strains from the Tohoku area.

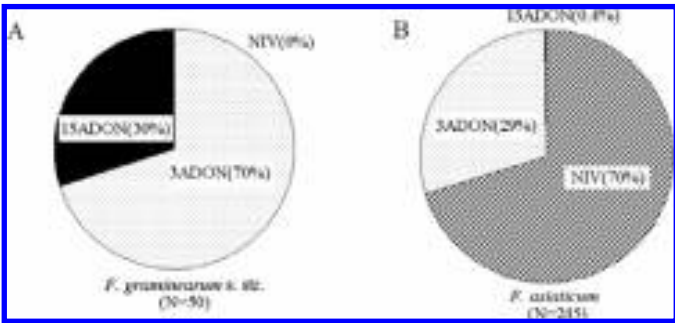


Fig. 5. Trichothecene chemotype compositions of Japanese strains of *Fusarium graminearum* s. str. (A) and *Fusarium asiaticum* (B). The chemotype of *F. graminearum* s. str. (n = 50) and *F. asiaticum* (n = 245) strains was determined by chemotype-specific multiplex polymerase chain reaction for *Tri3* and *Tri12* (28).

TABLE 4. Genome composition of field strains of *Fusarium graminearum* species complex

			PCR-RFLP marker ^b															
			Chromosome No. 1 ^c						No. 2					No. 3		No. 4		
Species	Strain information	No. of strains ^a	HK 703	HK 379	HK 249	HK 345	HK 463	HK 145	HK 731	HK 435	HK 517	HK 757	HK 253	HK 293	HK 337	HK 527	HK 271	
<i>F. graminearum</i> s. str. ^d	Japanese field strains	48	G ^e	G	G	G	G	G	G	G	G	G	G	G	G/U ^f	G	G	
<i>F. asiaticum</i> ^d	Japanese field strains	50	A ^g	A	A	A	A	A	A	A	A	A	A	A	A	A	A	
<i>F. graminearum</i> s. str.	Reference strains (NRRL31084 and NRRL34097)	2	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	
<i>F. asiaticum</i>	Reference strains (NRRL26156 and NRRL6101)	2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	

^a Strains from Table 2. All 33 strains from the Tohoku area are included.

^b *Hinf*I digestion of the polymerase chain reaction (PCR) amplicon displays polymorphism between *F. graminearum* s. str. and *F. asiaticum*.

^c Chromosome numbers are from Gale et al. (5).

^d Species were determined by diagnostic PCR-restriction fragment length polymorphism (RFLP) developed in this study.

^e "G" stands for *F. graminearum* s. str. pattern.

^f Strain 0223101 has a unique pattern ("U") for the HK337 marker.

^g "A" stands for *F. asiaticum* pattern.

Also, trichothenece chemotype composition was significantly different between *F. graminearum* s. str. and *F. asiaticum* (Fig. 5). These results strongly suggest that gene flow between these species is limited by some unknown factor(s) under natural conditions. Similarly, hybrids between *F. graminearum* s. str. and *F. cortaderiae* were not detected in New Zealand (13). Infrequent interspecific hybridization in nature is consistent with the fact that nine species could be resolved phylogenetically within the *F. graminearum* complex (17,18). Since a high level of segregation distortion was observed in three of the nine linkage groups in an in vitro cross between *F. graminearum* s. str. and *F. asiaticum* (7), interspecific hybridization may be maladaptive. It is worth considering that different timing of sexual reproduction of these species may restrict gene flow between them. However, absence of hybridization evidence in the field as shown in this study does not exclude occasional and low level of hybridization that may play a role in the dynamics of these populations. On the other hand, outcrossing within both species appears to be common as shown by population analyses (4,30). Additional studies are needed, especially within the Tohoku area, to determine what factors may restrict interspecific gene flow in Japan, and elsewhere.

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